(12) UK Patent Application (19) GB (11) 2 234 587 (13) A

(43) Date of A publication 06.02.1991.

(21) Application No 9016851.9

(22) Date of filing 01.08.1990

(30) Priority data
(31) 0199299 (32) 02.08.1989 (33) JP

(51) INT CL[5]
G01N 33/569

(52) UK CL (Edition K)
G1B BAD B103 B302
C6Y Y115 Y125 Y129 Y150 Y156 Y157 Y189 Y191 Y196
U1S S1351 S2419

(56) Documents cited
GB 2189317 A
EP 0135378 A1
WO 86/04352 A1
GB 2176891 A
EP 0017908 A1
WO 85/02685 A1
EP 0237384 A2
WO 87/06706 A1

(58) Field of search
UK CL (Edition K) C3H HF5, G1B BAD BAE

(71) Applicant
Chisso Corporation
(Incorporated in Japan)
6-32, Nakanoshima 3-chome, Kita-ku, Osaka-shi, Osaka 530, Japan

(72) Inventors
Masashi Ushiyama
Hiroaki Wakamoto
Hiroshi Morita

(74) Agent and/or Address for Service
Marks & Clerk
57-60 Lincoln's Inn Fields, London, WC2A 3LS, United Kingdom

(54) ELISA kit for detecting bacteria comprising polyclonal antibodies

(57) An ELISA kit for the detection of bacteria comprises polyclonal antibodies, which kit preferably comprises a carrier-bound antibody and a free antibody linked to an enzyme. The kit may be used to detect caliform bacteria, especially *Klebsiella* and *Enterobacter* spp. and *Salmonella* sp.

A KIT FOR DETECTING COLIFORM BACTERIA

The present invention relates to a kit for detecting coliform bacteria which is a hygienic indicator of food or drinking water.

The coliform bacteria are publicly defined as "all of Gram-negative and non spore-forming short rods which assimilate lactose producing gases and organic acids aerobically or facultative anaerobically". The genera *Escherichia* Family, *Klebsiella* and the like belong to the coliform bacteria. The presence of coliform bacteria in food or drinking water implies the possibility of the direct or indirect contact with feces and urine of men or animal and the contaminant. Accordingly, it is very important to detect the coliform bacteria in food sanitation.

Hitherto, for detecting coliform bacteria publicly, a brilliant green lactose bile broth (BGLB) or a lactose broth in which Darham tubes are inserted is used. A food sample is added to said medium and the medium is cultivated at 35-37°C for 24-48 hours. The accumulation of gas is observed in the Darham tube. The medium having the Darham tubes in which the accumulation of gas is observed should be submitted to confirmation by using an Endo medium and the like. It takes a long period of time, namely 48-72 hours, from the beginning of the cultivation to the end of the confirmation. For this reason, it is impossible to test a processing food before shipping or sanitary control of each process at a factory. There are not many simple methods using a paper strip and the like. However, these methods are uncertainty in absolute accuracy, and it takes 12 hours to cultivate bacteria. The test of the bacteria requires an equipment controlled under sterile conditions and a skillful personnel.

Kits applying enzyme-linked immunosorbent assay and a

DNA probe technique for detecting several kinds of colitis germs and salmonellae are commercially available, lately. These kits are more convenient than the above methods and it takes a shorter time for testing (about a half). However, since these kits are used for particularly detecting colitis germs and salmonellae in the individual case, they are not used for detecting all of the coliform bacteria.

For detecting the coliform bacteria, the following conditions are required. 1)Most of the coliform bacteria are detectable and 2)the test process is simple. Accordingly, the object of the present invention is to provide a method for detecting the coliform bacteria and a kit for applying the method satisfying those conditions.

The present invention resides in a kit for detecting coliform bacteria, comprising a carrier coated with a polyclonal antibody of an enterobacteriaceae strain and an enzyme-antibody conjugate. Preferably, the kit for detecting the coliform bacteria comprises a carrier coated with polyclonal antibody of cells belonging to enterobacteriaceae which is previously boiled, an enzyme labelled antibody having a marker enzyme conjugated the polyclonal antibody, a substrate of the marker enzyme and a chromogen.

Any kind of enterobacteriaceae strains can be used in the present invention. Preferably, *Enterobacter cloacae*, *Klebsiella pneumoniae* and *Salmonella typhimurium* can be used for obtaining antibodies having a cross reactivity in the coliform bacteria and the pathogenic bacteria belonging to enterobacteriaceae.

The polyclonal antibody is obtainable from blood serum of animals such as a rabbit, a guinea pig and a mouse after injecting the cells of enterobacteriaceae which is previously boiled. The antiserum obtained can be used as it is or after it is purified by a known method.

Two or more antisera can be mixed. As the carrier, a plastic test tube, a microtiter plate, a filter paper, a nitrocellulose membrane, plastic beads, glass beads, unwoven cloth or the like can be used. As the marker enzyme, horseradish peroxidase, alkaline phosphatase, β-galactosidase or the like can be used. As the substrate of peroxidase, hydrogen peroxide or urea peroxide can be used.

The antibody and the marker enzyme are conjugated by a known method such as a glutaraldehyde method, a periodic acid method, a maleimide method, a pyridyldisulfide method or the like. As the chromogen, ABTS (2,2'-azinodi(3-ethylbenzothiazoline)-6'-sulfonic acid), 5-aminosalicylic acid, luminol, TMB (3,3',5,5'-tetramethylbenzidine) or the like can be used. p-Nitrophenyl phosphoric acid as the substrate of alkaline phosphatase, and o- or p-nitrophenyl-β-D-galactoside or 4-methylumbelliferyl-β-D-galactoside as the substrate of β-galactosidase can be used.

The method for using the kit for detecting the coliform bacteria group is described more specifically in the following. The sample of food or drinking water is diluted in a proper quantity of a sterile physiological buffered saline, and the solution obtained is added to a portion of culture and incubated at 37°C for four hours or more. The incubated liquid is added to a carrier coated with a polyclonal antibody and incubated at a room temperature for 30 minutes. A suspension of previously boiled strains of *Bacillus subtilis* IFO 13719 is added to a carrier at the same time and incubated. Then, materials which are not adsorbed by coated antibodies are washed well several times in a buffer solution or the like. A solution of enzyme-antibody conjugate is added to the carrier and incubated at a room temperature for 30 minutes. The carrier is washed in a buffer solution or

the like, and a substrate or a substrate and a chromogen are added to the carrier and the culture solution is incubated at a room temperature. The same method as mentioned above is applied to a negative control. 30 minutes after, the reaction is stopped by adding dilute sulfuric acid and the absorbance or the fluorescence strength of the sample is determined. When the number of cells of coliform bacteria is 10^5/ml and more in the culture after the incubation, the absorbance or the fluorescence strength of the sample is proportionated to a logarithm of the number of the cells. When the absorbance or the fluorescence strength of the sample is 2.5 times or more of that of the negative control, the coliform bacteria group in the sample is judged to be positive.

According to the present invention, it provides a kit which can effectively detect almost of the coliform bacteria, the process is simple, the sensitivity of the kit response is quite good and the test is conducted in a short time.

The following examples illustrate the present invention more specifically, but these will not always be precise in practical application.

Example 1

Phosphate buffered saline pH 7.4 containing previously boiled strains of *Salmonella typhimurium* IFO 12529 was intraperitoneously injected to a rabbit once a week for three weeks, and it was intravenously injected to the rabbit once a week for three weeks. The blood was collected eight weeks after to obtain antiserum. 5 ml of the antiserum was diluted ten times with a solution of 0.5% Tween 80 in phosphate buffered saline, and 2 ml of the solution obtained was passed through 10 ml of a column (size: 1 cm diameter and 13 cm high) having a carrier of formylcellufine (manufactured by CHISSO CORPORATION,

trade name) to which lipopolysaccharide was bound. The column was washed with 30 ml of the same buffer solution, and anti-salmonella antibody was eluted with glycine-hydrochloride buffer pH 2.5. Tris-hydroxylamine was added to the elute to obtain a solution of pH 8.5. The solution was dialyzed overnight against phosphate buffered saline to obtain an anti-salmonella antibody solution.

Antibody solutions of *Enterobacter cloacae* IFO 13535 and *Klebsiella pneumoniae* JCM 1662 were obtained by using the above method.

One part portions of these antibody solutions were mixed. 1 ml of the mixture was diluted 500 times with 0.01 M phosphate buffer pH 7.2, 100 µl portions of the solution obtained was pipetted in each well of a micro-titer plate. After the plate was left at 37°C for one hour, the antibody solution was discarded. Then, 0.01 M sodium carbonate buffer of pH 7.5 containing 3% egg albumin was added in an amount of 300 µl per well. After the plate was left at 37°C for one hour, the contents were discarded, and the plate was washed with 0.01 M phosphate buffer of pH 7.0 for three times and swished water off. The plate was freeze-dried, put in a vinyl bag having a drying agent and stored at 4°C. The plate was stable for at least six months in the above conditions.

The other hand, 40 mg of anti-salmonella antibody described above was mixed with a solution of 30 mg of horseradish peroxidase having an EIA grade in 5 ml of 0.1 M sodium carbonate buffer (pH 7.0). Slowly stirring the solution obtained with a magnetic stirrer, 50 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was added little by little and reacted for 20 hours at a room temperature while adjusting to pH 6.0 - 7.0 with 0.1 N hydrochloric acid and 0.1 N sodium hydroxide. The reaction solution was passed through a column of cellufine GCL-1000sf (manufactured by CHISSO CORPORATION, trade

name) to separate and remove unreactive materials. The solution obtained was an enzyme labelled anti-salmonella antibody solution.

As the same method, solutions of enzyme labelled anti-enterobacter and anti-klebsiella antibodies were prepared.

Furthermore, *Escherichia coli* K12 IFO 3301, *Salmonella typhimurium* IFO 12529, *Enterobacter cloacae* IFO 13535, *Yersinia aldovae* JCM 5892, *Klebsiella pneumoniae* JCM 1662, *Aeromonas hydrophila subspecies hydrophilla* JCM 1027, *Serratia marcescens* JCM 1239 and *Proteus vulgaris* IFO 3851 in each amount of a platinum ear pick were incubated in a bouillon medium at 35°C for three hours. After the culture solution was immersed in hot water for five minutes and cooled, the solution was added in an amount of 100 µl per well of the microtiter plate which was previously prepared and incubated at 37°C. 30 minutes after, each well of the plate was washed with distilled water containing 0.05% Tween 20 for three times.

Then, one part portions of the enzyme labelled anti-salmonella antibody, the enzyme labelled anti-klebsiella antibody and the enzyme labelled anti-enterobacter antibody were mixed. The mixture was added to each well of the plate and incubated at 37°C. 30 minutes after, each well of the plate was washed with distilled water containing 0.05% Tween 20 for three times. After swishing water off, 0.2 ml of the mixture of ABTS (2,2'-azinodi (3-ethylbenzothiazolin)-6'-sulfonic acid) and citrate buffered solution pH 5.5 of 0.1 M hydrogen peroxide was added to each well and left for 15 minutes to develop color. The absorbance was determined at 405 nm with an immunoreader. The results are shown in Table 1.

Table 1

Strain	Absorbance at 405 nm*
Escherichia coli K12 IFO 3301	0.86
Salmonella typhimurium IFO 12529	0.85
Enterobacter cloacae IFO 13535	0.74
Yersinia aldovae JCM 5892	0.90
Klebsiella pneumoniae JCM 1662	0.80
Aeromonas hydrophila sp. *hydrophilla* JCM 1027	0.05
Serratia marcescens JCM 1239	0.10
Proteus vulgaris IFO 3851	0.03

* Absorbance 0.2 and more is judged to be positive.
Absorbance of the negative control is 0.08.

As shown in Table 1, the color development of *Salmonella*, *Escherichia*, *Yersinia*, *Enterobacter* and *Klebsiella* were positive, and the color development of *Aeromonas*, *Serratia* and *Proteus* were negative. The results are preferable because the latter strains do not belong to the coliform bacteria. Strictly speaking, although *Salmonella* and *Yersinia* do not belong to the coliform bacteria, these strains caused poisoning from eating. Accordingly, the detection results of these strains are preferable.

Example 2

Escherichia coli K12 in an amount of a platinum ear pick was added to 50 g of minced meat. The mixture was diluted with 200 ml of physiological saline to obtain suspension. 1 ml, 0.1 ml and 0.01 ml of suspensions, and 1 ml, 0.1 ml and 0.01 ml of dilute solutions diluted with physiological saline to 10^3 times and 10^6 times were inoculated with 10 ml of BGLB cultures having five Darham fermentation tubes, and with 10 ml of BGLB cultures having no Darham fermentation tubes, respectively.

The BGLB cultures having Darham fermentation tubes were incubated at 37°C for 24 hours and 48 hours and the production of gas was tested. The results are shown in Table 2 as a comparative example.

Table 2

Number of Darham fermentation tubes wherein gas was produced			
	Amount of sample (ml)		
Dilution	1	0.1	0.01
x 1	5	5	5
x 10^3	5	5	3
x 10^6	2	0	0

The BGLB cultures having no fermentation tubes were incubated at 37°C for 5 hours and each sample were deter-

mined by enzyme-linked immunosorbent assay (ELISA) with antibody-adsorbed plates and marker enzyme solutions prepared by the method described in Example 1. The results are shown in Table 3.

Table 3

Number of test tubes wherein positive is observed by ELISA			
	Amount of sample (ml)		
Dilution	1	0.1	0.01
x 1	5	5	5
x 10^3	5	5	5
x 10^6	5	3	1

As shown in Table 2 and 3, the samples of Example 2 are highly sensitive to those of the comparative example. The time required is 48 hours in the comparative example. The other hand, the time required is 7 hours in Example 2. The latter time is very shortened.

M&C FOLIO: 230P61726 WANGDOC: 0409D

CLAIMS

1. An ELISA kit for noxious, coliform or food-poisoning bacteria wherein the antibodies are polyclonal.

2. A kit according to claim 1, comprising antibody bound on a carrier, and free antibody linked to a marker enzyme.

3. A kit according to claim 1 or 2, wherein the polyclonal antibody has been raised against Salmonella, Klebsiella and/or Enterobacter spp.

4. A kit according to claim 3, wherein said bacterial species are Salmonella typhimurium, Klebsiella pneumoniae and/or Enterobacter cloacae.

5. A kit for detecting coliform bacteria, characterised in that the kit comprises a carrier coated with a polyclonal antibody of an enterobacteriaceae strain and a compound having a marker enzyme bound to the antibody, said kit optionally further comprising any one or more suitable features as defined in any preceding claim, in any suitable combination.

6. A kit according to claim 5, wherein the coliform bacteria comprise Salmonella typhimurium, Klebsiella pneumoniae and Enterobacter cloacae.

7. A kit for detecting bacteria and comprising polyclonal antibodies, substantially as hereinbefore described, with particular reference to the accompanying examples.

Published 1991 at The Patent Office, State House, 66/71 High Holborn, London WC1R 4TP. Further copies may be obtained from Sales Branch, Unit 6, Nine Mile Point, Cwmfelinfach, Cross Keys, Newport, NP1 7HZ. Printed by Multiplex techniques ltd, St Mary Cray, Kent